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Effects of Selected Hydrazines on the Early Death Rates of *Enterobacter cloacae*

S. A. London, C. R. Mantel, J. D. Robinson, and S. Luking

Toxic Hazards Division, Air Force Aerospace Medical Research Laboratory, Wright-Patterson AFB, OH 45433

The toxicity of hydrazine and several of its methylated derivatives has been studied in a variety of biological systems. London (1979) utilized a soil bacterium to compare the toxicities of these compounds and to suggest the validity of prokaryote and other simple systems as an adjunct to traditional toxicological techniques. Subsequent efforts (MANTEL and LONDON, 1980) were devoted to elucidating the mechanisms by which the hydrazines exert a toxic effect. The measurements used in these studies were concerned with growth kinetics, i.e., time and concentration parameters describing the growth cycle. The quantitation of growth was accomplished by turbidimetric determinations of cell mass which is an integrative description of particle size and number. This method provided useful information but was not sufficiently sensitive at the extremes of cell culture density. The insensitivity of low cell densities precluded ascertaining the effects of hydrazine exposure immediately after transfer and during the lag phase of the growth cycle.

Since the major indication of intoxication at the test concentrations used [10 ppm hydrazine (Hz); 20 ppm monomethylhydrazine (MMH); and 50 ppm 1,1-dimethylhydrazine (UDMH)] was an extension of the lag period, a possible mechanism of action is a random or selective killing of inoculum cells, the lengthening of the lag phase being inversely proportional to the fraction of inoculum cells killed (or prevented from initiating cell division). Since the experiments based on turbidimetric data could not address this aspect, we studied the early death rate kinetics of hydrazine-exposed cultures using a standard viable cell counting procedure as a more reliable quantitative method to enumerate cell death rate at low culture concentrations.

MATERIALS AND METHODS

The organism used in this study - Enterobacter cloacae strain D-31 - and the media, chemicals, and growth conditions were described in a previous report (MANTEL and LONDON, 1980). All experiments were conducted in Bellco 500 ml Nephelo culture flasks containing 100 ml of mineral salts medium (SMS) supplemented (when indicated) with 2 g/L glucose. Serum bottle

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stoppers were used in place of screw caps on the side-arm ports to enable sampling with a syringe.

The inocula were obtained from 16 ± 1 hr cultures incubated at 20 ± 1 C on a reciprocating shaker (100 oscill/min). The culture densities were adjusted with SMS to a turbidity of 40% transmittance and the flasks inoculated with 0.1 or 0.2 ml through the rubber serum stoppers. The inocula were not washed since the dilution factors of 500 or 1000 reduced carry-over of any residual nutrients to an insignificant level. In some experiments the '0' time count was obtained immediately after the inoculation of the flask and prior to the addition of the hydrazine compound; in other experiments the '0' time was determined by counting the inoculum culture prior to addition to the Nephelo flasks.

At the indicated times a 0.25 ml aliquot was removed aseptically from the appropriate flask, transferred to a sterile 13 x 100 mm test tube covered with a Morton cap, and vigorously agitated for approximately 20 sec with a Vortex® mixer. The largest total volume of culture removed during any experiment was 3.5 ml (0.25 ml x 14 samples) or 3.5% which did not alter significantly the surface area/volume ratio of medium in the flask. Two separate dilution series were prepared by delivering 0.1 ml volumes with a sterile MLA pipettor (LONDON, 1977) into 9.9 ml Tryptic Soy Broth (Difco) dilution tubes, agitating for 20 sec and diluting further as required. Aliquots of 0.1 ml were dispensed in triplicate with the MLA pipettor on the surface of Plate Count Agar (Difco) and spread with a flamed, alcohol- sterilized glass L-rod. (Tests indicated adherence to the glass rod did not significantly affect counts.) Colonies were counted after 24 hr incubation at 20 ± 1 C with the aid of a Quebec colony counter. Electronic counting devices did not prove satisfactory since at colony densities of approximately 100/plate contiguous colonies could not be discriminated. The counts reported in this study, obtained at different dilutions, are the average of 6 determinations (3 replicates x 2 dilution series) and are all presented as colony forming units (cfu) x 10^4 or 10^6/ml for comparison purposes. Five experiments were conducted with variations in sampling time and concentration of hydrazines. When higher concentrations of hydrazines were used, the elevated pH of the medium was adjusted to 7.1 with sterile dilute HCL.

RESULTS AND DISCUSSION

The viable count data for one of the five experiments conducted are presented in Table 1. Two flasks were prepared for each experimental condition and counts obtained from each flask. The data describe the effects of the compounds at the concentrations used in previous studies (10 ppm Hz, 20 ppm MMH, and 50 ppm UDMH) in both a growth (glucose present) and non-growth environment. The variation in the counts at "0" time is due to the inaccuracy of delivering the inoculum with a tuberculin syringe and the tendency for D-31 to form clumps. In addition, the

TABLE 1. The Effect of Hydrazines on the Growth and Viability of D-31 as Determined by Viable Count

Time	C + G		C - G		H + G		H - G		M + G		M - G		U + G		U - G	
Hr.	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
0	5.68	2.77	4.22	2.35	7.03	1.50	2.23	2.44	6.87	2.57	4.34	2.80	9.89	2.57	13.8	2.45
1.5	0.67	0.10	0.57	0.42	1.10	0.35	0.87	0.89	0.67	0.32	0.88	0.52	0.52	0.21	0.90	0.40
3	1.7	0.30	0.24	0.13	1.84	0.70	1.00	1.20	0.50	0.19	0.74	0.15	1.00	0.10	0.72	0.22
5	19.4	1.17	0.44	0.13	1.95	0.97	2.02	1.88	0.95	0.32	0.49	0.10	8.5	0.06	0.59	0.09
7	154.	30.	0.25	0.14	2.42	0.90	2.37	1.10	1.02	0.99	0.47	0.15	47.	12.0	0.35	0.15
11	1017.	575.	0.37	0.07	2.15	1.10	1.55	2.02	1.15	3.85	0.77	0.34	767.	247.	0.24	0.12
15	1447.	620.	0.30	0.10	3.30	1.15	2.17	1.75	29.	5.0	1.14	0.24	1512.	495.	0.42	0.37
20	1467.	792.	0.50	0.15	1030.	364.	2.77	1.23	215.	95.0	0.98	0.28	1979.	640.	0.58	0.27
25	1819.	1985.	0.26	0.26	-	-	3.19	3.30	455.	762.	1.56	0.88	1269.	1488.	0.38	0.30
29	1813.	1545.	0.37	0.29	1347.	1000.	2.99	2.85	545.	557.	1.44	0.96	1760.	1579.	0.31	0.40
38	1782.	1552.	0.30	0.28	1680.	1885.	1.84	3.53	563.	592.	1.04	0.90	1322.	1388.	0.42	0.59
50	1872.	1625.	0.33	0.37	2240.	2570.	1.74	3.49	875.	759.	1.64	1.32	1738.	1243.	0.59	0.95
62	1314.	1970.	0.18	0.49	2600.	2750.	1.97	2.02	739.	852.	1.35	1.15	1552.	1985.	0.49	1.06
74	1850.	1710.	0.52	0.49	2840.	1990.	0.80	1.44	854.	879.	1.37	1.08	1690.	1820.	0.38	1.08

Values are cfu x 10^6/ml and are averages of six determinations. All flasks were inoculated at the same (sequential) time from one 16 hr culture. A and B refer to duplicate flasks.

C = Control; H = Hydrazine; M = MMH; U = UDMH; +G = SMS with Glucose, -G = SMS without Glucose

TABLE 2. The Effect of Hydrazines on Viable Cell Count of D-31 Cultures

Time	Control		Hydrazine (ppm)								MMH (ppm)								UDMH (ppm)							
Min.	0	0G	10	10G	20	20G	50	50G	100	100G	10	10G	20	20G	50	50G	100	100G	50	50G	100	100G	500	500G	10	10 G
0	86	94	99	94	106	117	126	102	129	96	107	108	99	105	193	110	140	105	211	185	141	177	145	175	130	163
5	102	90	123	102	105	121	138	108	150	108	118	107	-	110	184	112	167	132	220	162	127	186	143	231	172	179
10	94	81	115	97	95	90	122	105	126	110	106	99	104	106	197	109	176	130	174	134	99	158	121	168	173	177
15	91	71	113	101	108	95	124	93	135	117	105	94	113	101	153	99	179	132	202	121	103	143	100	119	160	156
30	78	69	98	83	105	94	119	101	133	104	97	96	108	78	160	97	183	130	150	108	80	121	96	100	159	164
45	81	66	104	103	102	87	147	106	126	110	83	90	91	91	143	108	162	125	116	103	88	101	73	81	144	117
60	85	70	97	107	112	94	126	101	145	98	71	92	95	90	131	113	164	138	116	103	91	96	81	119	157	134
75	97	67	106	107	109	88	114	104	124	89	64	74	94	87	126	110	169	127	83	104	98	80	80	85	144	126
90	81	58	123	103	118	93	123	110	114	74	75	61	84	82	122	109	172	112	106	117	97	95	86	100	157	120
105	87	64	102	103	115	102	127	100	128	69	74	66	90	86	117	107	170	104	100	98	84	88	78	95	147	154
120	88	68	116	101	117	99	131	101	120	51	63	62	90	99	137	117	170	110	102	99	76	78	68	85	166	123
150	98	66	118	104	119	95	122	99	114	45	77	-	95	103	107	122	159	121	117	--	86	143	74	71	139	119
180	78	92	103	98	133	92	107	66	104	26	69	59	81	101	101	96	141	116	94	57	73	220	71	83	161	156

Values are cfu x 10^4/ml and are averages of six determinations. Each column represents an individual experiment performed on a separate day with a new 16 hr inoculum. G indicates glucose-containing medium.

difference in cell concentration at the initiation of the experiment resulted in turn in a difference in the ratio:amount of hydrazine compound/bacterial cell. However, the responses shown are representative of all the experiments conducted and indicate the effects of the hydrazines quite clearly:

1. A decrease in viable cell count of approximately the same magnitude occurred in all cultures, including controls, within the first sampling period.

2. Replicate cultures responded similarly although absolute numbers differed.

3. In the absence of glucose, viable cell counts remained relatively constant after the initial decrease.

Table 2 presents the results of an experiment in which four concentrations of each of the three hydrazine compounds were studied over a time period of three hours. Since previous experiments showed the decreases in viable cell count to occur shortly after transfer, samples were obtained at 5, 15, and 30 min intervals to define more clearly the shape of the "decay" curve. With the exception of a lessened decrease in viable count subsequent to transfer, these data agree in general with the previous four experiments. The values obtained at the shorter time periods indicate the observed decrease in viable count does not occur immediately after transfer but gradually over the first 1 to 2 hours of incubation. Significant cell death did not appear to occur in most cases, except as noted below, even at the highest concentrations of the hydrazines studied. The data for UDMH exposure suggest a dose-response relationship does not occur with this compound as indicated by comparison of the counts obtained at 180 min with the initial "0" time counts. This also applies to the MMH data; however, viability studies over longer time periods (see Tables 3 and 4 and LONDON, 1979) show a dose-response relationship. The data presented in Tables 3 and 4

Table 3. Viability of D-31 in a Growth Medium in the Presence of a Hydrazine Compound

Concentration (ppm)	Time to Reach Maximum Growth, Days		
	Hz	MMH	UDMH
10	2	1	ND
20	3	1	ND
50	10	2	1
100	- after 7	- after 10	1
500	ND	ND	1
1000	ND	ND	1

- = No Growth or Viability; ND = Not Determined

were obtained from the cultures established for the experiment summarized in Table 2. The flasks were maintained for up to 20 days after the initial 3 hr observation period and observed or sampled as indicated. Table 3 indicates that D-31, in a growth-promoting medium, initiated growth in the presence of the highest UDMH concentration studied (1000 ppm) to approximately the same extent and in the same time as the control. Growth in the presence of Hz was retarded, the inhibition exhibiting a dose-response relationship. At 100 ppm the culture was non-viable after 7 days. With 100 ppm MMH, the culture was non-viable in 10 days.

TABLE 4. Viable Count of D-31 in a Non-Growth Medium in the Presence of a Hydrazine Compound

Concentration (ppm)		Time in Days 1	2	4	5	6	7	8	9	10	11	12	13	17	18	19	20
0							TN	TN		TN			TN				TN
Hz	10					46	75		71			500				433	
	20				40	43		17			161				1		
	50			173	94		49			25				1			
	100			0	0		1			0				0			
MMH	10				TN												
	20				TN												
	50			400													
	100			200													
UDMH	50		TN			TN	TN										
	100		TN			55	0										
	500	TN			0	0											
	1000	TN			0	0											

TN = Too Numerous to Count
Values are cfu/0.1 ml

The data shown in Table 4 attest to both a dose-response and a difference in the toxicity of the three compounds. Thus, the difference in growth and survivability of exposed cells when compared to the control cultures can be observed more readily with increased incubation time.

The reduction in viability of D-31 in the first 60-90 min of exposure is compared in Table 5 for the three hydrazines. These data are a summary of all the experiments performed. Although some variation in procedure occurred and the first observation period was not the same (either 60 or 90 min), the means of these values suggest that 1) UDMH initiated a somewhat increased reduction in viability, 2) Hz exposure presented the smallest reduction in culture viability, and 3) viability may have been greater in a non-growth (no glucose) environment.

Table 5. The Effect of Hydrazines on the Viability of D-31 Inocula in Growth and Non-Growth Environments

Experiment Number	% Reduction in 1 or 1.5 hr							
	C + G	C - G	H + G	H - G	M + G	M - G	U + G	U - G
1	61	81	19	56	-	-	-	-
2	40	37	-	-	69	50	84	45
3A	89	87	85	61	91	80	95	94
3B	97	83	77	64	88	81	92	84
4	79	82	57	50	78	66	78	81
5	39	6	0	0	20	16	37	50
Average	67.5	62.7	47.6	46.2	69.2	58.6	77.2	70.8
SD	24.8	33.4	36.9	26.4	28.8	26.9	23.5	21.9

C = Control; H = Hydrazine; M = MMH; U = UDMH; +G = SMS with Glucose; -G = SMS without Glucose

The viability data for the concentrations of hydrazines studied previously (10 ppm Hz, 20 ppm MMH, and 50 ppm UDMH) are plotted in Fig. 1 for ease of comparison. With the exception of the rapid decrease in cfu in the first 30-45 min in the presence of UDMH, all growth curves were essentially flat, indicating viability of inoculum cells was not affected by any of the compounds. The slope of the curve for the control (unexposed) culture with glucose suggests these cells may have experienced a greater response to "transfer shock" than exposed cells.

The effects of hydrazines on the duration of the lag phase of growth of E. cloacae str D-31 provide a quantitative indication of the response of this organism to low levels of these compounds. Once lag growth is initiated, exposed cultures exhibit growth rates essentially identical to unexposed cells. Previous studies (LONDON and MANTEL, 1983; MANTEL and LONDON, 1980; LONDON, 1979) have shown this response to be both dose and compound related and possibly attributable to rate-controlling phenomena associated with transport mechanisms. The data presented in this report show that the extension of the lag phase is not a manifestation of a reduction in the number of inoculum cells. Although initial decreases in cell count in UDMH exposed cultures were greater than in control cultures, the growth rates, i.e., length of the lag periods, were not affected. By contrast, Hz exposure appeared to decrease the initial cell "die-off" as compared to the control cultures but eventually resulted in a significant delay in the onset of lag growth. With the exception of the early reduction (30-45 min) in UDMH cultures, cell counts remained relatively constant in both growth and non-growth environments (Fig. 1) for the 180 min observation period. The % reduction data (Table 5) suggest that cultures in minimal medium containing glucose exhibited a slightly larger decline in viable cell count, indicating that cells transferred to a growth promoting environment may be more sensitive to "transfer shock."

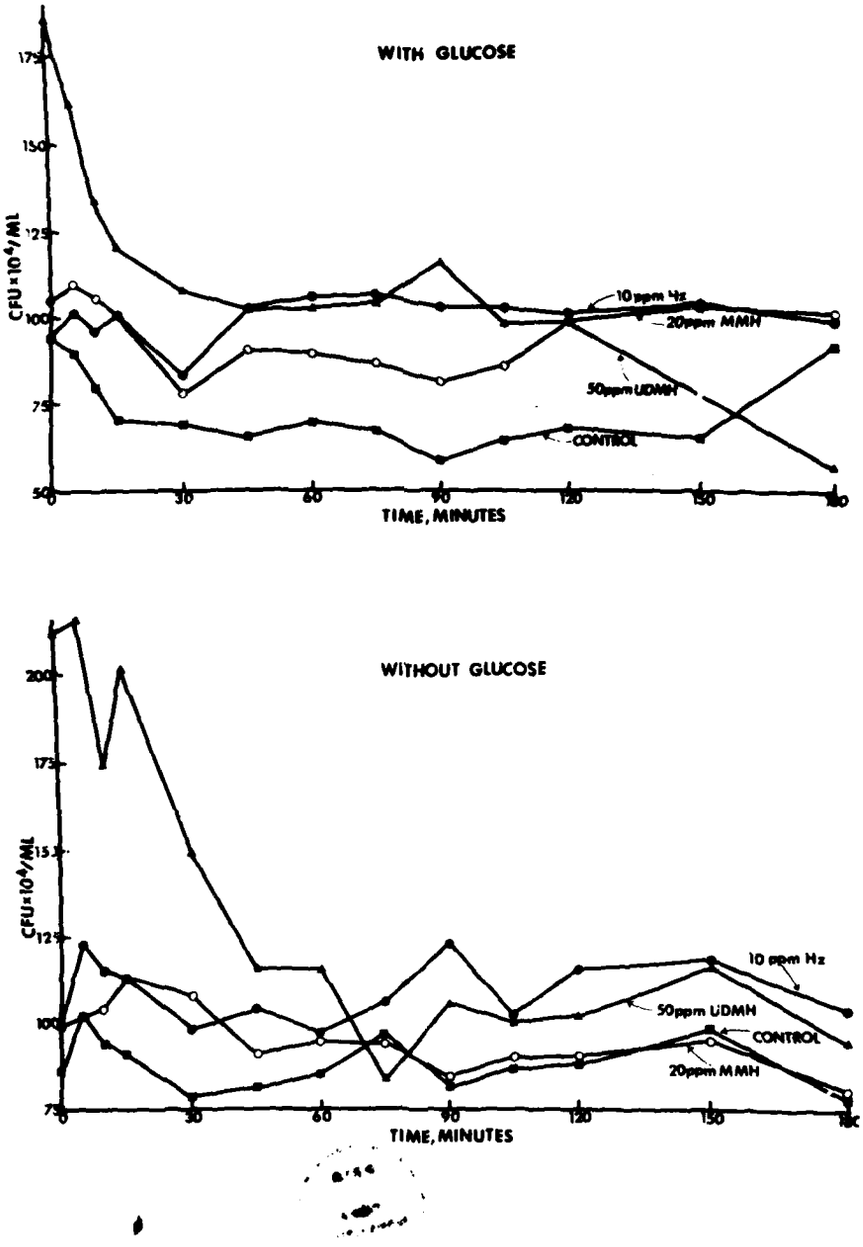

Figure 1. Viability of D-31 Inocula Exposed to Various Hydrazines

Longer incubation periods in a non-growth medium allow expression of concentration dependent cell death (Table 4). Higher concentrations of Hz and MMH extended the duration of the lag phase and caused cell death in glucose-free media. At 100 ppm both compounds prevented D-31 from initiating growth, indicating mechanisms other than transient interference with transport activities were operable at the higher concentration.

Substances that exert a bacteriostatic effect on particular species of bacteria do so by virtue of interfering with various synthetic mechanisms - DNA synthesis, RNA synthesis, cell wall synthesis, etc. Cells maintained in such inhibitory environments will ultimately become non-viable; however, removal to an inhibitor-free environment can result in the resumption of growth. In a complete medium containing a carbon and energy source (glucose), the toxicity of low concentrations of Hz and MMH to D-31 is expressed as a temporary inhibition. Cell death does not occur but rather a transient interference with cell replication processes is observed. These observations are consistent with previous findings that such processes could be associated with rate-controlling activities, i.e., transport mechanisms. The use of other probes that are known to affect cell membrane permeability may serve to elucidate this phenomenon further.

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